



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<p>(51) International Patent Classification ⁵ : C12N 15/00, C07K 7/10, 13/00 A61K 37/02, 27/00</p>	A1	<p>(11) International Publication Number: WO 92/07073 (43) International Publication Date: 30 April 1992 (30.04.92)</p>
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<p>(54) Title: OSTEOGENIC PEPTIDES</p> <p>(57) Abstract</p> <p>Disclosed are 1) the cDNA and amino acid sequences for novel polypeptide chains useful as subunits of dimeric osteogen- ic proteins, 2) osteogenic devices comprising these proteins in association with an appropriate carrier matrix, 3) methods of prod- ucing the polypeptide chains using recombinant DNA technology, and 4) methods of using the osteogenic devices to mimic the natural course of endochondral bone formation in mammals.</p>		

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Osteogenic peptides

Background of the Invention

5 This invention relates to novel polypeptide chains
and to osteogenic proteins comprising these polypeptide
chains which are capable of inducing osteogenesis in
mammals; to genes encoding the polypeptide chains; to
10 methods for their production using recombinant DNA
techniques, and to bone and cartilage repair procedures
using the osteogenic proteins.

 Mammalian bone tissue is known to contain one or
more proteinaceous materials, presumably active during
15 growth and natural bone healing, which can induce a
developmental cascade of cellular events resulting in
endochondral bone formation. This active factor (or
factors) has variously been referred to in the
literature as bone morphogenetic or morphogenic
20 protein, bone inductive protein, osteogenic protein,
osteogenin, or osteoinductive protein.

 The developmental cascade of bone differentiation
consists of recruitment of mesenchymal cells,
25 proliferation of progenitor cells, calcification of
cartilage, vascular invasion, bone formation,
remodeling, and finally marrow differentiation (Reddi
(1981) Collagen Rel. Res. 1:209-226).

30 Though the precise mechanisms underlying these
phenotypic transformations are unclear, it has been
shown that the natural endochondral bone
differentiation activity of bone matrix can be
dissociatively extracted and reconstituted with

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inactive residual collagenous matrix to restore full bone induction activity (Sampath and Reddi, (1981) Proc. Natl. Acad. Sci. USA 78:7599-7603). This provides an experimental method for assaying protein
05 extracts for their ability to induce endochondral bone in vivo. Several species of mammals produce closely related protein as demonstrated by cross species implant experiments (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595).

10 The potential utility of these proteins has been recognized widely. It is contemplated that the availability of the protein would revolutionize orthopedic medicine, certain types of plastic surgery, and various periodontal and craniofacial reconstructive
15 procedures.

The observed properties of these protein fractions have induced an intense research effort in various laboratories directed to isolating and identifying the pure factor or factors responsible for osteogenic
20 activity. The current state of the art of purification of osteogenic protein from mammalian bone is disclosed by Sampath et al. ((1987) Proc. Natl. Acad. Sci. USA 84: 7109-7113). Urist et al. (1984) Proc. Soc. Exp. Biol. Med. 173: 194-199 disclose a human osteogenic
25 protein fraction which was extracted from demineralized cortical bone by means of a calcium chloride-urea inorganic-organic solvent mixture, and retrieved by differential precipitation in guanidine-hydrochloride and preparative gel electrophoresis. The authors
30 report that the protein fraction has an amino acid composition of an acidic polypeptide and a molecular weight in a range of 17-18 kD.

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Urist et al. (1984) Proc. Natl. Acad. Sci. USA 81: 371-375 disclose a bovine bone morphogenetic protein extract having the properties of an acidic polypeptide and a molecular weight of approximately 18 kD. The 05 authors reported that the protein was present in a fraction separated by hydroxyapatite chromatography, and that it induced bone formation in mouse hindquarter muscle and bone regeneration in trephine defects in rat and dog skulls. Their method of obtaining the extract 10 from bone results in ill-defined and impure preparations.

European Patent Application Serial No. 148,155, published October 7, 1985, purports to disclose osteogenic proteins derived from bovine, porcine, and 15 human origin. One of the proteins, designated by the inventors as a P3 protein having a molecular weight of 22-24 kD, is said to have been purified to an essentially homogeneous state. This material is reported to induce bone formation when implanted into 20 animals.

International Application No. PCT/087/01537, published January 14, 1988, discloses an impure fraction from bovine bone which has bone induction qualities. The named applicants also disclose putative 25 "bone inductive factors" produced by recombinant DNA techniques. Four DNA sequences were retrieved from human or bovine genomic or cDNA libraries and expressed in recombinant host cells. While the applicants stated that the expressed proteins may be bone morphogenic 30 proteins, bone induction was not demonstrated, suggesting that the recombinant proteins are not osteogenic. The same group reported subsequently (Science, 242:1528, Dec, 1988) that three of the four

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factors induce cartilage formation, and postulate that bone formation activity "is due to a mixture of regulatory molecules" and that "bone formation is most likely controlled ... by the interaction of these
05 molecules." Again, no bone induction was attributed to the products of expression of the cDNAs. See also Urist et al., EP0,212,474 entitled Bone Morphogenic Agents.

Wang et al. (1988) Proc. Nat. Acad. Sci. USA 85:
10 9484-9488 discloses the purification of a bovine bone morphogenetic protein from guanidine extracts of demineralized bone having cartilage and bone formation activity as a basic protein corresponding to a molecular weight of 30 kD determined from gel elution.
15 Purification of the protein yielded proteins of 30, 18 and 16 kD which, upon separation, were inactive. In view of this result, the authors acknowledged that the exact identity of the active material had not been determined.

20 Wang et al. (1990) Proc. Nat. Acad. Sci. USA 87: 2220-2227 describes the expression and partial purification of one of the cDNA sequences described in PCT 87/01537. Consistent cartilage and/or bone formation with their protein requires a minimum of 600
25 ng of 50% pure material.

International Application No. PCT/89/04458 published April 19, 1990 (Int. Pub. No. WO90/003733), describes the purification and analysis of a family of osteogenic factors called "P3 OF 31-34". The protein
30 family contains at least four proteins, which are characterized by peptide fragment sequences. The impure mixture P3 OF 31-34 is assayed for osteogenic

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05 activity. The activity of the individual proteins is
neither assessed nor discussed.

10 It is an object of this invention to provide novel
polypeptide chains useful as subunits of dimeric
osteogenic proteins capable of endochondral bone
formation in allogenic and xenogenic implants in
mammals, including humans. Another object is to
provide genes encoding these polypeptide chains and
methods for the production of osteogenic proteins
15 comprising these polypeptide chains using recombinant
DNA techniques, as well as to provide antibodies
capable of binding specifically to these proteins.

These and other objects and features of the
invention will be apparent from the description,
drawings, and claims which follow.

Summary of the Invention

This invention provides novel polypeptide chains useful as either one or both subunits of dimeric osteogenic proteins which, when implanted in a mammalian body in association with a matrix, can induce at the locus of the implant the full developmental cascade of endochondral bone formation and bone marrow differentiation.

A key to these developments was the elucidation of amino acid sequence and structure data of native bovine osteogenic protein. A protocol was developed which results in retrieval of active, substantially pure osteogenic protein from bovine bone having a half-maximum bone forming activity of about 0.8 to 1.0 ng per mg of implant. The availability of the material enabled the inventors to elucidate key structural details of the protein necessary to achieve bone formation. Knowledge of the protein's amino acid sequence and other structural features enabled the identification and cloning of native genes in the human genome.

Consensus DNA sequences based on partial sequence data and observed homologies with regulatory proteins disclosed in the literature were used as probes for extracting genes encoding osteogenic protein from human genomic and cDNA libraries. One of the consensus sequences was used to isolate a previously unidentified gene which, when expressed, encoded a protein comprising a region capable of inducing endochondral bone formation when properly modified, incorporated in a suitable matrix, and implanted as disclosed herein. The gene, called "hOP1" or "OP-1" (human OP-1), is

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described in greater detail in copending U.S. 422,699, the disclosure of which is herein incorporated by reference.

In its native form, hOP1 expression yields an
05 immature translation product ("hOP1-PP", where "PP"
refers to "prepro form") of about 400 amino acids that
subsequently is processed to yield a mature sequence of
139 amino acids ("OP1-18"). The active region
(functional domain) of the protein comprises the
10 C-terminal 97 amino acids of the hOP1 sequence ("OPS").
A long active sequence is OP7 (comprising the
C-terminal 102 amino acids).

Further probing of mammalian cDNA libraries (human
and mouse) with sequences specific to hOP1 also has
15 identified novel OP1-like sequences herein referred to
as "OP2" ("hOP2" or "mOP2"). The OP2 proteins share
significant amino acid sequence homology, approximately
74%, with the active region of the OP1 proteins (e.g.,
OP7), and less homology with the intact mature form
20 (e.g., OP1-18, 58% amino acid homology).

The amino acid sequence of the osteogenic proteins
disclosed herein also share significant homology with
various of the regulatory proteins on which the
consensus probe was modeled. In particular, the
25 proteins share significant homology in their C-terminal
sequences, which comprise the active region of the
osteogenic proteins. (Compare, for example, OP7 with
DPP from *Drosophila* and Vgl from *Xenopus*. See, for
example, U.S. Pat. No. 5,011,691). In addition, these
30 proteins share a conserved six or seven cysteine
skeleton in this region (e.g., the linear arrangement
of these C-terminal cysteine residues is conserved in

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the different proteins.) See, for example, OP7, whose sequence defines the seven cysteine skeleton, or OPS, whose sequence defines the six cysteine skeleton. The OP2 proteins also contain an additional cysteine residue within this region.

Thus, in one preferred aspect, the invention comprises osteogenic proteins comprising a polypeptide chain comprising an amino acid sequence described by Seq. ID No. 3 or 5, including allelic and species variants thereof, and naturally-occurring or biosynthetic mutants, such that a dimeric protein comprising this polypeptide chain has a conformation capable of inducing endochondral bone formation when implanted in a mammal in association with a suitable matrix. Useful proteins include the full-length protein, mature proteins and truncated proteins comprising the functional domain described by the C-terminal.

In addition, the invention is not limited to these specific constructs. Thus, the osteogenic proteins of this invention comprising any of these polypeptide chains may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology which may be naturally occurring or biosynthetically derived, and active truncated or mutated forms of the native amino acid sequence, produced by expression of recombinant DNA in procaryotic or eucaryotic host cells. Active squances useful as osteogenic proteins of this invention are envisioned to include proteins capable of inducing endochondral bone formation when implanted in a mammal in association wiht a matrix and having at lest a 70% sequence homology, preferably at

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least 80%, with the amino acid sequence of OPS. This includes longer forms of a given protein, as well as allelic variants and muteins, including addition and deletion mutants, such as those which may alter the conserved C-terminal cysteine skeleton, provided that the alteration still allows the protein to form a dimeric species having a conformation capable of inducing bone formation in a mammal when implanted in the mammal in association with a matrix.

The novel polypeptide chains and the osteogenic proteins they comprise can be expressed from intact or truncated cDNA or from synthetic DNAs in procaryotic or eucaryotic host cells, and then purified, cleaved, refolded, dimerized, and implanted in experimental animals. Currently preferred host cells include E.coli or mammalian cells, such as CHO, COS or BSC cells. The osteogenic protein of the invention may include forms having varying glycosylation patterns, varying N-termini, a family of related proteins having regions of amino acid sequence homology, and active truncated or mutated forms of native or biosynthetic proteins, produced by expression of recombinant DNA in host cells.

Thus, in view of this disclosure, skilled genetic engineers can isolate genes from cDNA or genomic libraries of various different species which encode appropriate amino acid sequences, or construct DNAs from oligonucleotides, and then can express them in various types of host cells, including both procaryotes and eucaryotes, to produce large quantities of active proteins capable of inducing bone formation in mammals including humans. In view of this disclosure, those skilled in the art, using standard immunology

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techniques also may create antibodies capable of binding specifically to the osteogenic proteins disclosed herein, including fragments thereof.

The osteogenic proteins are useful in clinical applications in conjunction with a suitable delivery or support system (matrix). The matrix is made up of particles of porous materials. The pores must be of a dimension to permit progenitor cell migration and subsequent differentiation and proliferation. The particle size should be within the range of 70 - 850 mm, preferably 150mm - 420mm. It may be fabricated by close packing particulate material into a shape spanning the bone defect, or by otherwise structuring as desired a material that is biocompatible (non-inflammatory) and, biodegradable in vivo to serve as a "temporary scaffold" and substratum for recruitment of migratory progenitor cells, and as a base for their subsequent anchoring and proliferation. Currently preferred carriers include particulate, demineralized, guanidine extracted, species-specific (allogenic) bone, and specially treated particulate, protein extracted, demineralized, xenogenic bone. Optionally, such xenogenic bone powder matrices also may be treated with proteases such as trypsin and/or fibril modifying agents to increase the intraparticle intrusion volume and surface area. Useful agents include solvents such as dichloromethane, trichloroacetic acid, acetonitrile and acids such as trifluoroacetic acid and hydrogen fluoride. Alternatively, the matrix may be treated with a hot aqueous medium having a temperature within the range of about 37°C to 75°C, including a heated acidic aqueous medium. Other potentially useful matrix materials comprise collagen, homopolymers and copolymers of glycolic acid and lactic acid,

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hydroxyapatite, tricalcium phosphate and other calcium phosphates.

05 The osteogenic proteins and implantable osteogenic devices enabled and disclosed herein will permit the physician to obtain optimal predictable bone formation to correct, for example, acquired and congenital craniofacial and other skeletal or dental anomalies (Glowacki et al. (1981) Lancet 1:959-963). The devices may be used to induce local endochondral bone formation
10 in non-union fractures as demonstrated in animal tests, and in other clinical applications including dental and periodontal applications where bone formation is required. Another potential clinical application is in cartilage repair, for example, in the treatment of
15 osteoarthritis.

Brief Description of the Drawing

The foregoing and other objects of this invention, the various features thereof, as well as the invention itself, may be more fully understood from the following
20 description, when read together with the accompanying drawings, in which:

FIGURE 1 compares the amino acid sequences of the mature mOP-2 and hOP-2 polypeptide chains: hOP2-A and mOP2-A; and

25 FIGURE 2 compares the amino acid sequences of the mature OP1 and OP2 polypeptide chains: OP1-18, mOP1-S, hOP2-A and mOP2-A.

Description

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Purification protocols first were developed which enabled isolation of the osteogenic protein present in crude protein extracts from mammalian bone. (See PCT WO 89/09787, published 19-OCT-89, and U.S. Serial No. 05 179,406 filed April 8, 1988, now U.S. Patent No. 4,968,950). The development of the procedure, coupled with the availability of fresh calf bone, enabled isolation of substantially pure bovine osteogenic protein (bOP). bOP was characterized significantly; 10 its ability to induce cartilage and ultimately endochondral bone growth in cat, rabbit, and rat were demonstrated and studied; it was shown to be able to induce the full developmental cascade of bone formation previously ascribed to unknown protein or proteins in 15 heterogeneous bone extracts. This dose dependent and highly specific activity was present whether or not the protein was glycosylated (see Sampath et al., (1990) J. Biol. Chem. 265: 13198-13205). Sequence data obtained from the bovine materials suggested probe designs which 20 were used to isolate human genes. The OP human counterpart proteins have now been expressed and extensively characterized.

These discoveries enabled preparation of DNAs encoding totally novel, non-native protein constructs 25 which individually as homodimers and combined with other species as heterodimers are capable of producing true endochondral bone (see PCT WO 09788, published 19-OCT-89, and US Serial No. 315,342, filed 23-FEB-89, now U.S. Patent No. 5,011,691). They also permitted 30 expression of the natural material, truncated forms, muteins, analogs, fusion proteins, and various other variants and constructs, from cDNAs and genomic DNAs retrieved from natural sources or from synthetic DNA produced using the techniques disclosed herein and

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using automated, commercially available equipment. The DNAs may be expressed using well established molecular biology and recombinant DNA techniques in procaryotic or eucaryotic host cells, and may be oxidized and
05 refolded in vitro if necessary, to produce biologically active protein.

One of the DNA sequences isolated from human genomic and cDNA libraries encoded a previously unidentified gene, referred to herein as OP1. The
10 protein encoded by the isolated DNA was identified originally by amino acid homology with proteins in the TGF- β family. Consensus splice signals were found where amino acid homologies ended, designating exon-intron boundaries. Three exons were combined to obtain
15 a functional TGF- β -like domain containing seven cysteines. (See, for example, U.S. Patent No. 5,011,691, or Ozkaynak, E. et al., (1990) EMBO. 9: 2085-2093).

The full-length cDNA sequence for hOP1, and its
20 encoded "prepro" form "hOP1-PP," which includes an N-terminal signal peptide sequence, are disclosed in Seq. ID No. 1 (residues 1-431). The mature form of the hOP1 protein expressed in mammalian cells, "OP1-18", is described by amino acid residues 293-431 of Seq. ID
25 No. 1. The full length form of hOP1, as well as various truncated forms of the gene, and fused genes, have been expressed in E. coli and numerous mammalian cells (see, for example, published PCT application WO 91/05802, published 2-MAY-91) and all have been shown
30 to have osteogenic activity when implanted in a mammal in association with a suitable matrix.

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Given the foregoing amino acid and DNA sequence information, various nucleic acids (RNAs and DNAs) can be constructed which encode at least the active region of the hOP1 protein (e.g., OPS or OP7) and various
05 analogs thereof (including allelic and species variants and those containing genetically engineered mutations), as well as fusion proteins, truncated forms of the mature proteins, and similar constructs. Moreover, DNA
10 hybridization probes can be constructed from fragments of the hOP1 DNA or designed de novo based on the hOP1 DNA or amino acid sequence. These probes then can be used to screen different genomic and cDNA libraries to identify additional osteogenic proteins.

The DNAs can be produced by those skilled in the
15 art using well known DNA manipulation techniques involving genomic and cDNA isolation, construction of synthetic DNA from synthesized oligonucleotides, and cassette mutagenesis techniques. 15-100mer
20 oligonucleotides may be synthesized on a Biosearch DNA Model 8600 Synthesizer, and purified by polyacrylamide gel electrophoresis (PAGE) in Tris-Borate-EDTA buffer. The DNA then may be electroeluted from the gel. Overlapping oligomers may be phosphorylated by T4
25 polynucleotide kinase and ligated into larger blocks which may also be purified by PAGE.

DNAs used as hybridization probes may be labelled (e.g., as with a radioisotope, by nick-translation) and used to identify clones in a given library containing
30 DNA to which the probe hybridizes, following techniques well known in the art. The libraries may be obtained commercially or they may be constructed de novo using conventional molecular biology techniques. Further information on DNA library construction and

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hybridization techniques can be found in numerous texts known to those skilled in the art. See, for example, F.M. Ausubel., ed., Current Protocols in Molecular Biology-Vol. 1, (1989). In particular, see unit 5,
05 "Construction of Recombinant DNA Libraries" and Unit 6, "Screening of Recombinant Libraries."

The DNA from appropriately identified clones then can be isolated, subcloned (preferably into an expression vector), and sequenced. Plasmids containing
10 sequences of interest then can be transfected into an appropriate host cell for protein expression and further characterization. The host may be a procaryotic or eucaryotic cell since the former's inability to glycosylate protein will not destroy the
15 protein's osteogenic activity. Useful host cells include E. coli, Saccharomyces, the insect/baculovirus cell system, myeloma cells, and various mammalian cells. The vector additionally may encode various sequences to promote correct expression of the
20 recombinant protein, including transcription promoter and termination sequences, enhancer sequences, preferred ribosome binding site sequences, preferred mRNA leader sequences, preferred signal sequences for protein secretion, and the like. The DNA sequence
25 encoding the gene of interest also may be manipulated to remove potentially inhibiting sequences or to minimize unwanted secondary structure formation. The recombinant osteogenic protein also may be expressed as a fusion protein. After being translated, the protein
30 may be purified from the cells themselves or recovered from the culture medium. All biologically active protein forms comprise dimeric species joined by disulfide bonds or otherwise associated, produced by oxidizing and refolding one or more of the various

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recombinant polypeptide chains within an appropriate eucaryotic cell or in vitro after expression of individual subunits. A detailed description of osteogenic protein expressed from recombinant DNA in E. coli is disclosed in U.S. Serial No. 660,162, filed 27-FEB-91, the disclosure of which incorporated by reference herein. A detailed description of osteogenic protein expressed from recombinant DNA in numerous different mammalian cells is disclosed in PCT WO91/05802, also incorporated herein by reference.

Finally, in view of the disclosure made herein, and using standard methodologies known in the art, persons skilled in the art can raise polyclonal and monoclonal antibodies against all or part of a polypeptide chain disclosed herein, such that the antibodies are capable of binding specifically to an epitope on the polypeptide chain. Useful protocols can be found in, for example, Molecular Cloning-A Laboratory Manual (Sambrook et al. eds., Cold Spring Harbor Press 2nd ed. 1989). See Book 3, Section 18.

Exemplification

In an effort to identify additional DNA sequences encoding osteogenic proteins, a hybridization probe specific to the C-terminus of the DNA of mature OP-1 was prepared using a StuI-EcoRI digest fragment of OP-1 (base pairs 1034-1354 in Sequence ID No. 1), and labelled with ^{32}P by nick translation, as described in the art. As disclosed supra, the OP1 C-terminus encodes a key functional domain, e.g., the "active region" for osteogenic activity. The C-terminus also is the region of the protein whose amino acid sequence shares specific amino acid sequence homology with

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particular proteins in the TGF- β super-family of regulatory proteins, and which includes the conserved cysteine skeleton.

Approximately 7×10^5 phages of an oligo(dT) primed
05 17.5 days p.c. mouse embryo 5' stretch cDNA (gt10)
library (Clonetech, Inc., Palo Alto, CA) was screened
with the labelled probe. The screen was performed
using the following stringent hybridization conditions:
40% formamide, 5 x SSPE, 5 x Denhart's solution, 0.1%
10 SDS, at 37°C overnight, and washing in 0.1 x SSPE, 0.1%
SDS at 50°C.

Five recombinant phages were purified over three
rounds of screening. Phage DNA was prepared from all
five phages, subjected to an EcoRI digest, subcloned
15 into the EcoRI site of a common pUC-type plasmid
modified to allow single strand sequencing, and
sequenced using means well known in the art.

Two different DNAs were identified by this
procedure. One DNA, referred to herein as mOP1, has
20 substantial homology to the mature form of OP1 (about
98%), and is described in detail in copending USSN
600,024, filed 18-Oct-90. A second DNA, encoding the
C-terminus of a related gene and referred to herein as
mOP2, also was identified by this procedure. The
25 N-terminus of the gene encoding mOP2 was identified
subsequently by screening a second mouse cDNA library
(Mouse PCC4 cDNA (ZAP) library, Stratagene, Inc., La
Jolla, CA).

Mouse OP2 (mOP2) protein shares significant amino
30 acid sequence homology with the amino acid sequence of
the hOP1 active region, e.g., OPS or OP7, about 74%

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homology, and less homology with the intact mature form, e.g., OP1-18, about 58% homology. The cDNA sequence, and the encoded amino acid sequence, for the full length mOP-2 protein is depicted in Sequence ID
05 No. 3. The full-length form of the protein is referred to as the prepro form of mOP-2 ("mOP2-PP"), and includes a signal peptide sequence at its N-terminus. The amino acid sequence Leu-Ala-Leu-Cys-Ala-Leu (amino acid residues 13-18 of Sequence ID No. 3) is believed
10 to constitute the cleavage site for the removal of the signal peptide sequence, leaving an intermediate form of the protein, the "pro" form, to be secreted from the expressing cell. The amino acid sequence Arg-Ala-Pro-Arg-Ala (amino acid residues 255-259 of Sequence ID
15 No. 3) is believed to constitute the cleavage site that produces the mature form of the protein, herein referred to as "mOP2-A", and described by residues 259-397 of Seq. ID No. 3. Residues 301-397 of Seq. ID
20 No. 3 correspond to the region defining the conserved six cysteine skeleton. Residues 296-397 of Seq. ID No. 3 correspond to the region defining the conserved seven cysteine skeleton.

Using a probe prepared from the pro region of mOP2 (an EcoRI-BamHI digest fragment, bp 467-771 of Sequence
25 ID No. 3), a human hippocampus library was screened (human hippocampus cDNA lambda (ZAP II library Stratagene, Inc., La Jolla, CA) following essentially the same procedure as for the mouse library screens. The procedure identified the N-terminus of a novel DNA
30 encoding an amino acid sequence having substantial homology with mOP2. The C-terminus of the gene subsequently was identified by probing a human genomic library (in lambda phage EMBL-3, Clontech, Inc., Palo Alto, CA) with a labelled fragment from the novel human

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DNA in hand. The novel polypeptide chain encoded by this DNA is referred to herein as hOP2 protein, and shares almost complete amino acid identity (about 92% amino acid sequence homology) with mOP2-A (see Fig. 1 and infra).

The cDNA sequence, and the encoded amino acid sequence, for the prepro form of hOP2, "hOP2-PP", is described in Sequence ID No. 5. This full-length form of the protein also includes a signal peptide sequence at its N-terminus. The amino acid sequence Leu-Ala-Leu-Cys-Ala-Leu (amino acid residues 13-18 of Sequence ID No. 5) is believed to constitute the cleavage site for the removal of the signal peptide sequence, leaving an intermediate form of the protein, the "pro" form, to be secreted from the expressing cell. The amino acid sequence Arg-Thr-Pro-Arg-Ala (amino acid residues 257-261 of Sequence ID No. 5) is believed to constitute the cleavage site that produces what is believed to be the mature form of the protein, herein referred to as hOP2-A" and described by residues 261-399 of Seq. ID No. 5.

Additional mature species of hOP2 thought to be active include truncated sequences, "hOP2-P" (described by residues 264-399 of Seq. ID No. 5) and "hOP2-R" (described by residues 267-399 of Seq. ID No. 5), and a slightly longer sequence ("hOP2-S", described by residues 240-399 of Seq. ID No. 5). Residues 303-399 of Seq. ID No. 5 correspond to the region defining the conserved six cysteine skeleton. Residues 297-399 of Seq. ID No. 5 correspond to the region defining the conserved seven cystein skeleton.

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It should be noted that the nucleic acid sequence encoding the N-terminus of the prepro form of both mOP2 and hOP2 is rich in guanidine and cytosine base pairs. As will be appreciated by those skilled in the art, sequencing such a "G-C rich" region can be problematic, due to stutter and/or band compression. Accordingly, the possibility of sequencing errors in this region can not be ruled out. However, the definitive amino acid sequence for these and other, similarly identified proteins can be determined readily by expressing the protein from recombinant DNA using, for example, any of the means disclosed herein, and sequencing the polypeptide chain by conventional peptide sequencing methods well known in the art.

Figure 1 compares the amino acid sequences of mature mOP2 and hOP2. Identity is indicated by three dots (...) in the mOP2 sequence. As is evident from the figure, the amino acid sequence homology between the mature forms of these two proteins is substantial (92% homology between the mature sequences, about 95% homology within the C-terminal active region (e.g., residues 38-139 or 42-139 of Fig. 1.)

Fig. 2 compares the amino acid sequences for the mature forms of all four species of OP1 and OP2 proteins. Here again, identity is indicated by three dots (...). Like the mOP2 protein, the hOP2 protein shares significant homology (about 74%) with the amino acid sequence defining the OP1 active region (OPS or OP7, residues 43-139 and 38-139, respectively, in Fig. 2), and less homology with OP1-18 (about 58% homology). Both OP2 proteins share the conserved seven cysteine skeleton seen in the OP1 proteins. In

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and wherein Xaa at res. 2 = (Lys or Arg); Xaa at res. 3 = (Lys or Arg); Xaa at res. 9 = (Ser or Arg); Xaa at res. 11 = (Arg or Gln); Xaa at res. 16 = (Gln or Leu); Xaa at res. 19 = (Ile or Val); Xaa at res. 23 = (Glu or Gln); Xaa at res. 26 = (Ala or Ser); Xaa at res. 35 = (Ala or Ser); Xaa at res. 39 = (Asn or Asp); Xaa at res. 41 = (Tyr or Cys); Xaa at res. 50 = (Val or Leu); Xaa at res. 52 = (Ser or Thr); Xaa at res. 56 = (Phe or Leu); Xaa at res. 57 = (Ile or Met); Xaa at res. 58 = (Asn or Lys); Xaa at res. 60 = (Glu, Asp or Asn); Xaa at res. 61 = (Thr, Ala or Val); Xaa at res. 65 = (Pro or Ala); Xaa at res. 71 = (Gln or Lys); Xaa at res. 73 = (Asn or Ser); Xaa at res. 75 = (Ile or Thr); Xaa at res. 80 = (Phe or Tyr); Xaa at res. 82 = (Asp or Ser); Xaa at res. 84 = (Ser or Asn); Xaa at res. 87 = (Ile or Asp); Xaa at res. 89 = (Lys or Arg); Xaa at res. 91 = (Tyr, Ala or His); and Xaa at res. 97 = (Arg or Lys).

20 The high degree of homology exhibited between the various OP1 and OP2 proteins suggests that the novel osteogenic proteins identified herein will purify essentially as OP1 does, or with only minor modifications of the protocols disclosed for OP1.

25 Similarly, the purified mOP1, mOP2, and hOP2 proteins are predicted to have an apparent molecular weight of about 18 kDa as reduced single subunits, and an apparent molecular weight of about 36 kDa as oxidized dimers, as determined by comparison with molecular

30 weight standards on an SDS-polyacrylamide electrophoresis gel. Unglycosylated dimers (e.g., proteins produced by recombinant expression in E. coli) are predicted to have an apparent molecular weight of about 27 kDa. There appears to be one potential N


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                35                40
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
  45                50                55
05 Xaa Xaa Xaa Xaa Xaa Cys Cys Xaa Xaa Xaa Xaa
                60                65
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                70                75
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
                80                85
10 Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys Xaa
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```

"OPX-8C" (Sequence ID No. 9 comprising additional five residues at the N-terminus, including a conserved cysteine residue):

```

15 Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
   1                5                10
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
   15                20
20 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa
   25                30
Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa
   35                40                45
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
   50                55
25 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
   60                65
Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
   70                75
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
   80                85
30 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys
   90                95
Xaa Cys Xaa

```

100

MATRIX PREPARATION

A. General Consideration of Matrix Properties

05 The currently preferred carrier material is a
xenogenic bone-derived particulate matrix treated as
disclosed herein. This carrier may be replaced by
either a biodegradable-synthetic or synthetic-inorganic
matrix (e.g., hydroxylapatite (HAP), collagen,
tricalcium phosphate or polylactic acid, polyglycolic
10 acid and various copolymers thereof.)

Studies have shown that surface charge, particle
size, the presence of mineral, and the methodology for
combining matrix and osteogenic protein all play a role
in achieving successful bone induction. Perturbation
15 of the charge by chemical modification abolishes the
inductive response. Particle size influences the
quantitative response of new bone; particles between
75 μm and 420 μm elicit the maximum response.
Contamination of the matrix with bone mineral will
20 inhibit bone formation. Most importantly, the
procedures used to formulate OP onto the matrix are
extremely sensitive to the physical and chemical state
of both the osteogenic protein and the matrix.

25 The sequential cellular reactions in the
interface of the bone matrix/osteogenic protein
implants are complex. The multistep cascade includes:
binding of fibrin and fibronectin to implanted matrix,
chemotaxis of cells, proliferation of fibroblasts,
differentiation into chondroblasts, cartilage

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formation, vascular invasion, bone formation, remodeling, and bone marrow differentiation.

05 A successful carrier for osteogenic protein must perform several important functions. It must bind osteogenic protein and act as a slow release delivery system, accommodate each step of the cellular response during bone development, and protect the osteogenic protein from nonspecific proteolysis. In addition, selected materials must be biocompatible in vivo and
10 preferably biodegradable; the carrier must act as a temporary scaffold until replaced completely by new bone. Polylactic acid (PLA), polyglycolic acid (PGA), and various combinations have different dissolution rates in vivo. In bones, the dissolution rates can
15 vary according to whether the implant is placed in cortical or trabecular bone.

Matrix geometry, particle size, the presence of surface charge, and the degree of both intra-and-inter-particle porosity are all important to successful
20 matrix performance. It is preferred to shape the matrix to the desired form of the new bone and to have dimensions which span non-union defects. Rat studies show that the new bone is formed essentially having the dimensions of the device implanted.

25 The matrix may comprise a shape-retaining solid made of loosely adhered particulate material, e.g., with collagen. It may also comprise a molded, porous solid, or simply an aggregation of close-packed particles held in place by surrounding tissue.
30 Masticated muscle or other tissue may also be used. Large allogenic bone implants can act as a carrier for the matrix if their marrow cavities are cleaned and

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packed with particle and the dispersed osteogenic protein.

The preferred matrix material, prepared from xenogenic bone and treated as disclosed herein, 05 produces an implantable material useful in a variety of clinical settings. In addition to its use as a matrix for bone formation in various orthopedic, periodontal, and reconstructive procedures, the matrix also may be used as a sustained release carrier, or as a 10 collagenous coating for implants. The matrix may be shaped as desired in anticipation of surgery or shaped by the physician or technician during surgery. Thus, the material may be used for topical, subcutaneous, intraperitoneal, or intramuscular implants; it may be 15 shaped to span a nonunion fracture or to fill a bone defect. In bone formation or conduction procedures, the material is slowly absorbed by the body and is replaced by bone in the shape of or very nearly the shape of the implant.

20 Various growth factors, hormones, enzymes, therapeutic compositions, antibiotics, and other body treating agents also may be absorbed onto the carrier material and will be released over time when implanted as the matrix material is slowly absorbed. Thus, 25 various known growth factors such as EGF, PDGF, IGF, FGF, TGF- α , and TGF- β may be released in vivo. The material can be used to release chemotherapeutic agents, insulin, enzymes, or enzyme inhibitors.

B. Bone-Derived Matrices

1. Preparation of Demineralized Bone

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Demineralized bone matrix, preferably bovine bone matrix, is prepared by previously published procedures (Sampath and Reddi (1983) Proc. Natl. Acad. Sci. USA 80:6591-6595). Bovine diaphyseal bones (age 05 1-10 days) are obtained from a local slaughterhouse and used fresh. The bones are stripped of muscle and fat, cleaned of periosteum, demarrowed by pressure with cold water, dipped in cold absolute ethanol, and stored at -20°C. They are then dried and fragmented by crushing 10 and pulverized in a large mill. Care is taken to prevent heating by using liquid nitrogen. The pulverized bone is milled to a particle size in the range of 70-850 μm , preferably 150-420 μm , and is defatted by two washes of approximately two hours 15 duration with three volumes of chloroform and methanol (3:1). The particulate bone is then washed with one volume of absolute ethanol and dried over one volume of anhydrous ether yielding defatted bone powder. The defatted bone powder is then demineralized by four 20 successive treatments with 10 volumes of 0.5 N HCl at 4°C for 40 min. Finally, neutralizing washes are done on the demineralized bone powder with a large volume of water.

2. Guanidine Extraction

25 Demineralized bone matrix thus prepared is extracted with 5 volumes of 4 M guanidine-HCl, 50mM Tris-HCl, pH 7.0 for 16 hr. at 4°C. The suspension is filtered. The insoluble material is collected and used to fabricate the matrix. The material is mostly 30 collagenous in nature. It is devoid of osteogenic or chondrogenic activity.

3. Matrix Treatments

The major component of all bone matrices is Type-I collagen. In addition to collagen, demineralized bone extracted as disclosed above
05 includes non-collagenous proteins which may account for 5% of its mass. In a xenogenic matrix, these noncollagenous components may present themselves as potent antigens, and may constitute immunogenic and/or
10 inhibitory components. These components also may inhibit osteogenesis in allogenic implants by interfering with the developmental cascade of bone differentiation. It has been discovered that treatment of the matrix particles with a collagen
15 fibril-modifying agent extracts potentially unwanted components from the matrix, and alters the surface structure of the matrix material. Useful agents include acids, organic solvents or heated aqueous media. Various treatments are described below. A
20 detailed physical analysis of the effect these fibril-modifying agents have on demineralized, guanidine-extracted bone collagen particles is disclosed in PCT WO 90/10018, published 7-SEP-90.

After contact with the fibril-modifying agent, the treated matrix is washed to remove any extracted
25 components, following a form of the procedure set forth below:

1. Suspend in TBS (Tris-buffered saline) 1g/200 ml and stir at 4°C for 2 hrs; or in 6 M urea, 50 mM Tris-HCl, 500 mM NaCl, pH 7.0 (UTBS) or water and
30 stir at room temperature (RT) for 30 minutes (sufficient time to neutralize the pH);

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2. Centrifuge and repeat wash step; and

3. Centrifuge; discard supernatant; water wash residue; and then lyophilize.

3.1 Acid Treatments

05 1. Trifluoroacetic acid.

Trifluoroacetic acid is a strong non-oxidizing acid that is a known swelling agent for proteins, and which modifies collagen fibrils.

10 Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are collected. These particles are extracted with various percentages (1.0% to 100%) of trifluoroacetic acid and water (v/v) at 0°C or room temperature for 1-2 hours with constant stirring. The treated matrix is
15 filtered, lyophilized, or washed with water/salt and then lyophilized.

2. Hydrogen Fluoride.

Like trifluoroacetic acid, hydrogen fluoride is a strong acid and swelling agent, and also is
20 capable of altering intraparticle surface structure. Hydrogen fluoride is also a known deglycosylating agent. As such, HF may function to increase the osteogenic activity of these matrices by removing the antigenic carbohydrate content of any glycoproteins
25 still associated with the matrix after guanidine extraction.

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Bovine bone residue prepared as described above is sieved, and particles of the appropriate size are collected. The sample is dried in vacuo over P_2O_5 , transferred to the reaction vessel and exposed to
05 anhydrous hydrogen fluoride (10-20 ml/g of matrix) by distillation onto the sample at $-70^\circ C$. The vessel is allowed to warm to $0^\circ C$ and the reaction mixture is stirred at this temperature for 120 minutes. After evaporation of the hydrogen fluoride in vacuo, the
10 residue is dried thoroughly in vacuo over KOH pellets to remove any remaining traces of acid. Extent of deglycosylation can be determined from carbohydrate analysis of matrix samples taken before and after treatment with hydrogen fluoride, after washing the
15 samples appropriately to remove non-covalently bound carbohydrates. SDS-extracted protein from HF-treated material is negative for carbohydrate as determined by Con A blotting.

The deglycosylated bone matrix is next washed
20 twice in TBS (Tris-buffered saline) or UTBS, water-washed, and then lyophilized.

Other acid treatments are envisioned in addition to HF and TFA. TFA is a currently preferred acidifying reagent in these treatments because of its
25 volatility. However, it is understood that other, potentially less caustic acids may be used, such as acetic or formic acid.

3.2 Solvent Treatment

1. Dichloromethane.

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Dichloromethane (DCM) is an organic solvent capable of denaturing proteins without affecting their primary structure. This swelling agent is a common reagent in automated peptide synthesis, and is used in 05 washing steps to remove components.

Bovine bone residue, prepared as described above, is sieved, and particles of the appropriate size are incubated in 100% DCM or, preferably, 99.9% DCM/0.1% TFA. The matrix is incubated with the 10 swelling agent for one or two hours at 0°C or at room temperature. Alternatively, the matrix is treated with the agent at least three times with short washes (20 minutes each) with no incubation.

2. Acetonitrile.

15 Acetonitrile (ACN) is an organic solvent, capable of denaturing proteins without affecting their primary structure. It is a common reagent used in high-performance liquid chromatography, and is used to elute proteins from silica-based columns by perturbing 20 hydrophobic interactions.

Bovine bone residue particles of the appropriate size, prepared as described above, are treated with 100% ACN (1.0 g/30 ml) or, preferably, 99.9% ACN/0.1% TFA at room temperature for 1-2 hours 25 with constant stirring. The treated matrix is then water-washed, or washed with urea buffer, or 4 M NaCl and lyophilized. Alternatively, the ACN or ACN/TFA treated matrix may be lyophilized without wash.

3. Isopropanol.

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Isopropanol is also an organic solvent capable of denaturing proteins without affecting their primary structure. It is a common reagent used to elute proteins from silica HPLC columns.

05 Bovine bone residue particles of the appropriate size prepared as described above are treated with 100% isopropanol (1.0 g/30 ml) or, preferably, in the presence of 0.1% TFA, at room temperature for 1-2 hours with constant stirring. The
10 matrix is then water-washed or washed with urea buffer or 4 M NaCl before being lyophilized.

4. Chloroform

Chloroform also may be used to increase surface area of bone matrix like the reagents set forth
15 above, either alone or acidified.

Treatment as set forth above is effective to assure that the material is free of pathogens prior to implantation.

3.3 Heat Treatment

20 The currently most preferred agent is a heated aqueous fibril-modifying medium such as water, to increase the matrix particle surface area and porosity. The currently most preferred aqueous medium is an acidic aqueous medium having a pH of less than about
25 4.5, e.g., within the range of pH 2 - pH 4. which may help to "swell" the collagen before heating. 0.1% acetic acid, which has a pH of about 3, currently is preferred. 0.1 M acetic acid also may be used.

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Various amounts of delipidated, demineralized guanidine-extracted bone collagen are heated in the aqueous medium (1g matrix/30ml aqueous medium) under constant stirring in a water jacketed glass flask, and
05 maintained at a given temperature for a predetermined period of time. Preferred treatment times are about one hour, although exposure times of between about 0.5 to two hours appear acceptable. The temperature employed is held constant at a temperature generally
10 within the range of about 37°C to 75°C. The currently preferred heat treatment temperature is within the range of 45°C to 60°C.

After the heat treatment, the matrix is filtered, washed, lyophilized and used for implant. Where an
15 acidic aqueous medium is used, the matrix also is preferably neutralized prior to washing and lyophilization. A currently preferred neutralization buffer is a 200mM sodium phosphate buffer, pH 7.0. To neutralize the matrix, the matrix preferably first is
20 allowed to cool following thermal treatment, the acidic aqueous medium (e.g., 0.1% acetic acid) then is removed and replaced with the neutralization buffer and the matrix agitated for about 30 minutes. The neutralization buffer then may be removed and the
25 matrix washed and lyophilized (see infra).

The matrix also may be treated to remove contaminating heavy metals, such as by exposing the matrix to a metal ion chelator. For example, following thermal treatment with 0.1% acetic acid, the matrix may
30 be neutralized in a neutralization buffer containing EDTA (sodium ethylenediaminetetraacetic acid), e.g., 200 mM sodium phosphate, 5mM EDTA, pH 7.0. 5 mM EDTA provides about a 100-fold molar excess of chelator to

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residual heavy metals present in the most contaminated matrix tested to date. Subsequent washing of the matrix following neutralization appears to remove the bulk of the EDTA. EDTA treatment of matrix particles
05 reduces the residual heavy metal content of all metals tested (Sb, As, Be, Cd, Cr, Cu, Co, Pb, Hg, Ni, Se, Ag, Zn, Tl) to less than about 1 ppm. Bioassays with EDTA-treated matrices indicate that treatment with the metal ion chelator does not inhibit bone inducing activity.

10 The collagen matrix materials preferably take the form of a fine powder, insoluble in water, comprising nonadherent particles. It may be used simply by packing into the volume where new bone growth or sustained release is desired, held in place by
15 surrounding tissue. Alternatively, the powder may be encapsulated in, e.g., a gelatin or polylactic acid coating, which is adsorbed readily by the body. The powder may be shaped to a volume of given dimensions and held in that shape by interadhering the particles
20 using, for example, soluble, species-biocompatible collagen. The material may also be produced in sheet, rod, bead, or other macroscopic shapes.

Demineralized rat bone matrix used as an allogenic matrix in certain of the experiments
25 disclosed herein, is prepared from several of the dehydrated diaphyseal shafts of rat femur and tibia as described herein to produce a bone particle size which passes through a 420 μm sieve. The bone particles are subjected to dissociative extraction with 4 M
30 guanidine-HCl. Such treatment results in a complete loss of the inherent ability of the bone matrix to induce endochondral bone differentiation. The remaining insoluble material is used to fabricate the

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matrix. The material is mostly collagenous in nature, and upon implantation, does not induce cartilage and bone. All new preparations are tested for mineral content and osteogenic activity before use. The total
05 loss of biological activity of bone matrix is restored when an active osteoinductive protein fraction or a pure osteoinductive protein preparation is reconstituted with the biologically inactive insoluble collagenous matrix.

10

FABRICATION OF OSTEOGENIC DEVICE

The naturally sourced and recombinant protein as set forth above, and other constructs, can be combined and dispersed in a suitable matrix preparation using any of the methods described below. In general, 50-100
15 ng of active protein is combined with the inactive carrier matrix (e.g., 25 mg for rat bioassays). Greater amounts may be used for large implants.

1. Ethanol Precipitation

Matrix is added to osteogenic protein
20 dissolved in guanidine-HCl. Samples are vortexed and incubated at a low temperature (e.g., 4°C). Samples are then further vortexed. Cold absolute ethanol (5 volumes) is added to the mixture which is then stirred and incubated, preferably for 30 minutes at -20°C.
25 After centrifugation (microfuge, high speed) the supernatant is discarded. The reconstituted matrix is washed twice with cold concentrated ethanol in water (85% EtOH) and then lyophilized.

2. Acetonitrile Trifluoroacetic

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Acid Lyophilization

In this procedure, osteogenic protein in an acetonitrile trifluoroacetic acid (ACN/TFA) solution is added to the carrier material. Samples are vigorously
05 vortexed many times and then lyophilized. This method is currently preferred, and has been tested with osteogenic protein at varying concentrations and different levels of purity.

3. Urea Lyophilization

10 For those osteogenic proteins that are prepared in urea buffer, the protein is mixed with the matrix material, vortexed many times, and then lyophilized. The lyophilized material may be used "as is" for implants.

15 4. Buffered Saline Lyophilization

OP1 and OP2 preparations in physiological saline may also be vortexed with the matrix and lyophilized to produce osteogenically active material.

20 These procedures also can be used to adsorb other active therapeutic drugs, hormones, and various bioactive species to the matrix for sustained release purposes.

BIOASSAY

The functioning of the various proteins and
25 devices of this invention can be evaluated with an in vivo bioassay. Studies in rats show the osteogenic effect in an appropriate matrix to be dependent on the

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dose of osteogenic protein dispersed in the matrix. No activity is observed if the matrix is implanted alone. In vivo bioassays performed in the rat model also have shown that demineralized, guanidine-extracted xenogenic bone matrix materials of the type described in the literature are ineffective as a carrier, fail to induce bone, and produce an inflammatory and immunological response when implanted unless treated as disclosed above. In certain species (e.g., monkey) allogenic matrix materials also apparently are ineffective as carriers. The following sets forth various procedures for preparing osteogenic devices from the proteins and matrix materials prepared as set forth above, and for evaluating their osteogenic utility.

15 A. Rat Model

1. Implantation

The bioassay for bone induction as described by Sampath and Reddi ((1983) Proc. Natl. Acad. Sci. USA 80 6591-6595), herein incorporated by reference, may be used to monitor endochondral bone differentiation activity. This assay consists of implanting test samples in subcutaneous sites in recipient rats under ether anesthesia. Male Long-Evans rats, aged 28-32 days, were used. A vertical incision (1 cm) is made under sterile conditions in the skin over the thoracic region, and a pocket is prepared by blunt dissection. Approximately 25 mg of the test sample is implanted deep into the pocket and the incision is closed with a metallic skin clip. The day of implantation is designated as day one of the experiment. Implants were removed on day 12. The heterotropic site allows for the study of bone induction without the possible

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ambiguities resulting from the use of orthotropic sites. As disclosed herein, both allogenic (rat bone matrix) and xenogenic (bovine bone matrix) implants were assayed.

05 2. Cellular Events

Successful implants exhibit a controlled progression through the stages of protein-induced endochondral bone development, including: (1) transient infiltration by polymorphonuclear leukocytes on day
10 one; (2) mesenchymal cell migration and proliferation on days two and three; (3) chondrocyte appearance on days five and six; (4) cartilage matrix formation on day seven; (5) cartilage calcification on day eight; (6) vascular invasion, appearance of osteoblasts, and
15 formation of new bone on days nine and ten; (7) appearance of osteoblastic and bone remodeling and dissolution of the implanted matrix on days twelve to eighteen; and (8) hematopoietic bone marrow
20 differentiation in the ossicle on day twenty-one. The results show that the shape of the new bone conforms to the shape of the implanted matrix.

3. Histological Evaluation

Histological sectioning and staining is preferred to determine the extent of osteogenesis in
25 the implants. Implants are fixed in Bouins Solution, embedded in paraffin, and cut into 6-8 μ m sections. Staining with toluidine blue or hemotoxylin/eosin demonstrates clearly the ultimate development of endochondral bone. Twelve day implants are usually
30 sufficient to determine whether the implants contain newly induced bone.

4. Biological Markers

Alkaline phosphatase activity may be used as a marker for osteogenesis. The enzyme activity may be determined spectrophotometrically after homogenization
05 of the implant. The activity peaks at 9-10 days in vivo and thereafter slowly declines. Implants showing no bone development by histology have little or no alkaline phosphatase activity under these assay conditions. The assay is useful for quantitation and
10 obtaining an estimate of bone formation quickly after the implants are removed from the rat. Alternatively, the amount of bone formation can be determined by measuring the calcium content of the implant.

The invention may be embodied in other specific
15 forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather
20 than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced therein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 05 (i) APPLICANT: OPPERMAN, HERMANN
OZKAYNAK, ENGIN
KUBERASAMPATH, THANGAVEL
RUEGER, DAVID C.
- (ii) TITLE OF INVENTION: OSTEOGENIC DEVICES
- (iii) NUMBER OF SEQUENCES: 9
- 10 (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: TESTA, HURWITZ & THIBEALT
(B) STREET: 53 STATE STREET
(C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
15 (E) COUNTRY: U.S.A.
(F) ZIP: 02109
- (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
20 (C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:
- 25 (viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: PITCHER, EDMUND R.
(B) REGISTRATION NUMBER: 27,829
(C) REFERENCE/DOCKET NUMBER: CRRO56PC
- 30 (ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617/248-7000
(B) TELEFAX: 617/248-7100

(2) INFORMATION FOR SEQ ID NO:1:

- 35 (i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1822 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS

(F) TISSUE TYPE: HIPPOCAMPUS

05 (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 49..1341

(C) IDENTIFICATION METHOD: experimental

10 (D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"
 /product= "hOP1-PP"
 /evidence= EXPERIMENTAL
 /standard_name= "hOP1"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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	Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala Leu Trp Ala	
	5 10 15	
20	CCC CTG TTC CTG CTG CGC TCC GCC CTG GCC GAC TTC AGC CTG GAC AAC	153
	Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser Leu Asp Asn	
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	ATCCAATGAA CAAGATCCTA CAAGCTGTGC AGGCCAAAACC TAGCAGGAAA AAAAAACAAC	1591
	GCATAAAGAA AAATGGCCGG GCCAGGTCAT TGGCTGGGAA GTCTCAGCCA TGCACGGACT	1651
	CGTTTCCAGA GGTAATTATG AGCGCCTACC AGCCAGGCCA CCCAGCCGTG GGAGGAAGGG	1711
	GGCGTGGCAA GGGGTGGGCA CATTGGTGTC TGTGCGAAAG GAAAATTGAC CCGGAAGTTC	1771
30	CTGTAATAAA TGTCACAATA AAACGAATGA ATGAAAAAAA AAAAAAAAAA A	1822

(2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 431 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (ix) FEATURE:

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05 (D) OTHER INFORMATION: /Product="hOP1-PP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met His Val Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala
 1 5 10 15

10 Leu Trp Ala Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser
 20 25 30

Leu Asp Asn Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser
 35 40 45

Gln Glu Arg Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu
 50 55 60

15 Pro His Arg Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro
 65 70 75 80

Met Phe Met Leu Asp Leu Tyr Asn Ala Met Ala Val Glu Glu Gly Gly
 85 90 95

20 Gly Pro Gly Gly Gln Gly Phe Ser Tyr Pro Tyr Lys Ala Val Phe Ser
 100 105 110

Thr Gln Gly Pro Pro Leu Ala Ser Leu Gln Asp Ser His Phe Leu Thr
 115 120 125

Asp Ala Asp Met Val Met Ser Phe Val Asn Leu Val Glu His Asp Lys
 130 135 140

25 Glu Phe Phe His Pro Arg Tyr His His Arg Glu Phe Arg Phe Asp Leu
 145 150 155 160

Ser Lys Ile Pro Glu Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile
 165 170 175

30 Tyr Lys Asp Tyr Ile Arg Glu Arg Phe Asp Asn Glu Thr Phe Arg Ile
 180 185 190

Ser Val Tyr Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu
 195 200 205

Phe Leu Leu Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu
 210 215 220

Val Phe Asp Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg
 225 230 235 240

His Asn Leu Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser
 245 250 255

Ile Asn Pro Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn

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05          260          265          270
Lys Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe
      275          280          285
Arg Ser Ile Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser
      290          295          300
10 Lys Thr Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu
      305          310          315
Asn Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr
      325          330          335
15 Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu
      340          345          350
Gly Tyr Ala Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn
      355          360          365
Ser Tyr Met Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His
      370          375          380
20 Phe Ile Asn Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln
      385          390          395
Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile
      405          410          415
25 Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His
      420          425          430

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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1929 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (ix) FEATURE:
 - (A) NAME/KEY: CDS
 - (B) LOCATION: 103..1293
 - (D) OTHER INFORMATION: /function= "osteogenic protein"
 /product= "mOP2-PP"
 /note= "mOP2 cDNA"
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAATTCGCT GCCAGGCACA GGTGCGCCGT CTGGTCCTCC CCGTCTGGCG TCAGCCGAGC

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05	CCGACCAGCT ACCAGTGGAT GCGCGCCGGC TGAAAGTCCG AG ATG GCT ATG CGT	114
	Met Ala Met Arg	
	1	
10	CCC GGG CCA CTC TGG CTA TTG GGC CTT GCT CTG TGC GCG CTG GGA GGC	162
	Pro Gly Pro Leu Trp Leu Leu Gly Leu Ala Leu Cys Ala Leu Gly Gly	
	5 10 15 20	
	GGC CAC GGT CCC GGT CCC CCG CAC ACC TGT CCC CAG CGT CGC CTG GGA	210
	Gly His Gly Pro Gly Pro Pro His Thr Cys Pro Gln Arg Arg Leu Gly	
	25 30 35	
15	GCG CGC GAC CGG GAC ATG CAG CGT GAA ATC CTG CCG GTG CTC GGG CTA	258
	Ala Arg Asp Arg Asp Met Gln Arg Glu Ile Leu Pro Val Leu Gly Leu	
	40 45 50	
	CCG GGA CGC CCC GAC CCC GTG CAC AAC CCG CCG CTG CCC GGC ACG CAG	306
	Pro Gly Arg Pro Asp Pro Val His Asn Pro Pro Leu Pro Gly Thr Gln	
	55 60 65	
20	CGT GCG CCC CTC TTC ATG TTG GAC CTA TAC CAC GCC ATG ACC GAT GAC	354
	Arg Ala Pro Leu Phe Met Leu Asp Leu Tyr His Ala Met Thr Asp Asp	
	70 75 80	
25	GAC GAC GGC GGG CCA CCA CAG GCT CAC TTA GGC CGT GCC GAC CTG GTC	402
	Asp Asp Gly Gly Pro Pro Gln Ala His Leu Gly Arg Ala Asp Leu Val	
	85 90 95 100	
	ATG AGC TTC GTC AAC ATG GTG GAA CGC GAC CGT ACC CTG GGC TAC CAG	450
	Met Ser Phe Val Asn Met Val Glu Arg Asp Arg Thr Leu Gly Tyr Gln	
	105 110 115	
30	GAG CCA CAC TGG AAG GAA TTC CAC TTT GAC CTA ACC CAG ATC CCT GCT	498
	Glu Pro His Trp Lys Glu Phe His Phe Asp Leu Thr Gln Ile Pro Ala	
	120 125 130	
	GGG GAG GCT GTC ACA GCT GCT GAG TTC CGG ATC TAC AAA GAA CCC AGC	546
	Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Glu Pro Ser	
	135 140 145	
	ACC CAC CCG CTC AAC ACA ACC CTC CAC ATC AGC ATG TTC GAA GTG GTC	594
	Thr His Pro Leu Asn Thr Thr Leu His Ile Ser Met Phe Glu Val Val	
	150 155 160	
	CAA GAG CAC TCC AAC AGG GAG TCT GAC TTG TTC TTT TTG GAT CTT CAG	642
	Gln Glu His Ser Asn Arg Glu Ser Asp Leu Phe Phe Leu Asp Leu Gln	
	165 170 175 180	
	ACG CTC CGA TCT GGG GAC GAG GGC TGG CTG GTG CTG GAC ATC ACA GCA	690
	Thr Leu Arg Ser Gly Asp Glu Gly Trp Leu Val Leu Asp Ile Thr Ala	
	185 190 195	
	GCC AGT GAC CGA TGG CTG CTG AAC CAT CAC AAG GAC CTG GGA CTC CGC	738

05	Ala Ser Asp Arg Trp Leu Leu Asn His His Lys Asp Leu Gly Leu Arg 200 205 210	
	CTC TAT GTG GAA ACC GCG GAT GGG CAC AGC ATG GAT CCT GGC CTG GCT Leu Tyr Val Glu Thr Ala Asp Gly His Ser Met Asp Pro Gly Leu Ala 215 220 225	786
10	GGT CTG CTT GGA CGA CAA GCA CCA CGC TCC AGA CAG CCT TTC ATG GTA Gly Leu Leu Gly Arg Gln Ala Pro Arg Ser Arg Gln Pro Phe Met Val 230 235 240	834
	ACC TTC TTC AGG GCC AGC CAG AGT CCT GTG CGG GCC CCT CGG GCA GCG Thr Phe Phe Arg Ala Ser Gln Ser Pro Val Arg Ala Pro Arg Ala Ala 245 250 255 260	882
15	AGA CCA CTG AAG AGG AGG CAG CCA AAG AAA ACG AAC GAG CTT CCG CAC Arg Pro Leu Lys Arg Arg Gln Pro Lys Lys Thr Asn Glu Leu Pro His 265 270 275	930
20	CCC AAC AAA CTC CCA GGG ATC TTT GAT GAT GGC CAC GGT TCC CGC GGC Pro Asn Lys Leu Pro Gly Ile Phe Asp Asp Gly His Gly Ser Arg Gly 280 285 290	978
	AGA GAG GTT TGC CGC AGG CAT GAG CTC TAC GTC AGA TTC CGT GAC CTT Arg Glu Val Cys Arg Arg His Glu Leu Tyr Val Arg Phe Arg Asp Leu 295 300 305	1026
25	GGC TGG CTG GAC TGG GTC ATC GCC CCC CAG GGC TAC TCT GCC TAT TAC Gly Trp Leu Asp Trp Val Ile Ala Pro Gln Gly Tyr Ser Ala Tyr Tyr 310 315 320	1074
	TGT GAG GGG GAG TGT GCT TTC CCA CTG GAC TCC TGT ATG AAC GCC ACC Cys Glu Gly Glu Cys Ala Phe Pro Leu Asp Ser Cys Met Asn Ala Thr 325 330 335 340	1122
30	AAC CAT GCC ATC TTG CAG TCT CTG GTG CAC CTG ATG AAG CCA GAT GTT Asn His Ala Ile Leu Gln Ser Leu Val His Leu Met Lys Pro Asp Val 345 350 355	1170
	GTC CCC AAG GCA TGC TGT GCA CCC ACC AAA CTG AGT GCC ACC TCT GTG Val Pro Lys Ala Cys Cys Ala Pro Thr Lys Leu Ser Ala Thr Ser Val 360 365 370	1218
35	CTG TAC TAT GAC AGC AGC AAC AAT GTC ATC CTG CGT AAA CAC CGT AAC Leu Tyr Tyr Asp Ser Ser Asn Asn Val Ile Leu Arg Lys His Arg Asn 375 380 385	1266
	ATG GTG GTC AAG GCC TGT GGC TGC CAC TGAGGCCCGG CCCAGCATCC Met Val Val Lys Ala Cys Gly Cys His 390 395	1313
	TGCTTCTACT ACCTTACCAT CTGGCCGGGC CCCTCTCCAG AGGCAGAAAC CTTTCTATGT	1373

05 TATCATAGCT CAGACAGGGG CAATGGGAGG CCCTTCACTT CCCCTGGCCA CTCCTGCTA 1433
 AAATTCTGGT CTTTCCCAGT TCCTCTGTCC TTCATGGGGT TCGGGGGCTA TCACCCCGCC 1493
 CTCTCCATCC TCCTACCCCA AGCATAGACT GAATGCACAC AGCATCCCAG AGCTATGCTA 1553
 ACTGAGAGGT CTGGGGTCAG CACTGAAGGC CCACATGAGG AAGACTGATC CTTGGCCATC 1613
 CTCAGCCCAC AATGGCAAAT TCTGGATGGT CTAAGAAGCC CTGGAATTCT AAACCTAGATG 1673
 10 ATCTGGGCTC TCTGCACCAT TCATTGTGGC AGTTGGGACA TTTTATAGTA TAACAGACAC 1733
 ATACACTTAG ATCAATGCAT CGCTGTACTC CTTGAAATCA GAGCTAGCTT GTTAGAAAAA 1793
 GAATCAGAGC CAGGTATAGC GGTGCATGTC ATTAATCCCA GCGCTAAAGA GACAGAGACA 1853
 GGAGAATCTC TGTGAGTTCA AGGCCACATA GAAAGAGCCT GTCTCGGGAG CAGGAAAAAA 1913
 AAAAAAACG GAATTC 1929

15 (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 397 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

20 (ii) MOLECULE TYPE: protein

- (ix) FEATURE:
 - (D) OTHER INFORMATION: /Product= "mOP2-PP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

25 Met Ala Met Arg Pro Gly Pro Leu Trp Leu Leu Gly Leu Ala Leu Cys
 1 5 10 15
 Ala Leu Gly Gly Gly His Gly Pro Gly Pro Pro His Thr Cys Pro Gln
 20 25 30
 Arg Arg Leu Gly Ala Arg Asp Arg Asp Met Gln Arg Glu Ile Leu Pro
 35 40 45
 30 Val Leu Gly Leu Pro Gly Arg Pro Asp Pro Val His Asn Pro Pro Leu
 50 55 60
 Pro Gly Thr Gln Arg Ala Pro Leu Phe Met Leu Asp Leu Tyr His Ala
 65 70 75 80
 Met Thr Asp Asp Asp Asp Gly Gly Pro Pro Gln Ala His Leu Gly Arg
 85 90 95

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05 Ala Asp Leu Val Met Ser Phe Val Asn Met Val Glu Arg Asp Arg Thr
100 105 110
Leu Gly Tyr Gln Glu Pro His Trp Lys Glu Phe His Phe Asp Leu Thr
115 120 125
10 Gln Ile Pro Ala Gly Glu Ala Val Thr Ala Ala Glu Phe Arg Ile Tyr
130 135 140
Lys Glu Pro Ser Thr His Pro Leu Asn Thr Thr Leu His Ile Ser Met
145 150 155 160
Phe Glu Val Val Gln Glu His Ser Asn Arg Glu Ser Asp Leu Phe Phe
165 170 175
15 Leu Asp Leu Gln Thr Leu Arg Ser Gly Asp Glu Gly Trp Leu Val Leu
180 185 190
Asp Ile Thr Ala Ala Ser Asp Arg Trp Leu Leu Asn His His Lys Asp
195 200 205
20 Leu Gly Leu Arg Leu Tyr Val Glu Thr Ala Asp Gly His Ser Met Asp
210 215 220
Pro Gly Leu Ala Gly Leu Leu Gly Arg Gln Ala Pro Arg Ser Arg Gln
225 230 235 240
Pro Phe Met Val Thr Phe Phe Arg Ala Ser Gln Ser Pro Val Arg Ala
245 250 255
25 Pro Arg Ala Ala Arg Pro Leu Lys Arg Arg Gln Pro Lys Lys Thr Asn
260 265 270
Glu Leu Pro His Pro Asn Lys Leu Pro Gly Ile Phe Asp Asp Gly His
275 280 285
30 Gly Ser Arg Gly Arg Glu Val Cys Arg Arg His Glu Leu Tyr Val Arg
290 295 300
Phe Arg Asp Leu Gly Trp Leu Asp Trp Val Ile Ala Pro Gln Gly Tyr
305 310 315 320
Ser Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asp Ser Cys
325 330 335
35 Met Asn Ala Thr Asn His Ala Ile Leu Gln Ser Leu Val His Leu Met
340 345 350
Lys Pro Asp Val Val Pro Lys Ala Cys Cys Ala Pro Thr Lys Leu Ser
355 360 365
Ala Thr Ser Val Leu Tyr Tyr Asp Ser Ser Asn Asn Val Ile Leu Arg
370 375 380

05 Lys His Arg Asn Met Val Val Lys Ala Cys Gly Cys His
 385 390 395

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1941 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: HOMO SAPIENS
- (F) TISSUE TYPE: HIPPOCAMPUS

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 507..1703
- (D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"
 /product= "hOP2-PP"
 /note= "hOP2 (CDNA)"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

25	GGAATTCGG CCACAGTGGC GCCGGCAGAG CAGGAGTGGC TGGAGGAGCT GTGGTTGGAG	60
	CAGGAGGTGG CACGGCAGGG CTGGAGGGCT CCCTATGAGT GCGGAGACG GCCCAGGAGG	120
	CGCTGGAGCA ACAGCTCCA CACCGACCA AGCGGTGGCT GCAGGAGCTC GCCCATCGCC	180
	CCTGCGCTGC TCGGACCGCG GCCACAGCCG GACTGGCGGG TACGGCGGCG ACAGAGGCAT	240
	TGGCCGAGAG TCCCAGTCCG CAGAGTAGCC CCGGCCTCGA GCGGTGGCG TCCCGTCTCT	300
30	CTCCGTCCAG GAGCCAGGAC AGGTGTCGCG CGCGGGGCT CCAGGGACCG CGCCTGAGGC	360
	CGGCTGCCCG CCCGTCCCGC CCCGCCCGC CGCCCGCCG CCGCCGAGCC CAGCCTCCTT	420
	GCCGTCGGGG CGTCCCAGG CCCTGGGTCG GCCGCGGAGC CGATGCGCGC CGGCTGAGCG	480
	CCCCAGCTGA GCGCCCCCGG CCTGCC ATG ACC GCG CTC CCC GGC CCG CTC TGG	533
35	Met Thr Ala Leu Pro Gly Pro Leu Trp	
	1 5	
	CTC CTG GGC CTG GCG CTA TGC GCG CTG GGC GGG GGC GGC CCC GGC CTG	581
	Leu Leu Gly Leu Ala Leu Cys Ala Leu Gly Gly Gly Gly Pro Gly Leu	
	10 15 20 25	
	CGA CCC CCG CCC GGC TGT CCC CAG CGA CGT CTG GGC GCG CGC GAC CGG	629

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05	Arg Pro Pro Pro Gly Cys Pro Gln Arg Arg Leu Gly Ala Arg Asp Arg 30 35 40	
	GAC GTG CAG CGC GAG ATC CTG GCG GTG CTC GGG CTG CCT GGG CGG CCC Asp Val Gln Arg Glu Ile Leu Ala Val Leu Gly Leu Pro Gly Arg Pro 45 50 55	677
10	CGG CCC CGC GCG CCA CCC GCC GCC TCC CGG CTG CCC GCG TCC GCG CCG Arg Pro Arg Ala Pro Pro Ala Ala Ser Arg Leu Pro Ala Ser Ala Pro 60 65 70	725
15	CTC TTC ATG CTG GAC CTG TAC CAC CGC ATG GCC GGC GAC GAC GAC GAG Leu Phe Met Leu Asp Leu Tyr His Arg Met Ala Gly Asp Asp Asp Glu 75 80 85	773
	GAC GGC GCC GCG GAG GCC CTG GGC CGC GCC GAC CTG GTC ATG AGC TTC Asp Gly Ala Ala Glu Ala Leu Gly Arg Ala Asp Leu Val Met Ser Phe 90 95 100 105	821
20	GTT AAC ATG GTG GAG CGA GAC CGT GCC CTG GGC CAC CAG GAG CCC CAT Val Asn Met Val Glu Arg Asp Arg Ala Leu Gly His Gln Glu Pro His 110 115 120	869
	TGG AAG GAG TTC CGC TTT GAC CTG ACC CAG ATC CCG GCT GGG GAG GCG Trp Lys Glu Phe Arg Phe Asp Leu Thr Gln Ile Pro Ala Gly Glu Ala 125 130 135	917
25	GTC ACA GCT GCG GAG TTC CGG ATT TAC AAG GTG CCC AGC ATC CAC CTG Val Thr Ala Ala Glu Phe Arg Ile Tyr Lys Val Pro Ser Ile His Leu 140 145 150	965
30	CTC AAC AGG ACC CTC CAC GTC AGC ATG TTC CAG GTG GTC CAG GAG CAG Leu Asn Arg Thr Leu His Val Ser Met Phe Gln Val Val Gln Glu Gln 155 160 165	1013
	TCC AAC AGG GAG TCT GAC TTG TTC TTT TTG GAT CTT CAG ACG CTC CGA Ser Asn Arg Glu Ser Asp Leu Phe Phe Leu Asp Leu Gln Thr Leu Arg 170 175 180 185	1061
35	GCT GGA GAC GAG GGC TGG CTG GTG CTG GAT GTC ACA GCA GCC AGT GAC Ala Gly Asp Glu Gly Trp Leu Val Leu Asp Val Thr Ala Ala Ser Asp 190 195 200	1109
	TGC TGG TTG CTG AAG CGT CAC AAG GAC CTG GGA CTC CGC CTC TAT GTG Cys Trp Leu Leu Lys Arg His Lys Asp Leu Gly Leu Arg Leu Tyr Val 205 210 215	1157
40	GAG ACT GAG GAC GGG CAC AGC GTG GAT CCT GGC CTG GCC GGC CTG CTG Glu Thr Glu Asp Gly His Ser Val Asp Pro Gly Leu Ala Gly Leu Leu 220 225 230	1205
	GGT CAA CGG GCC CCA CGC TCC CAA CAG CCT TTC GTG GTC ACT TTC TTC Gly Gln Arg Ala Pro Arg Ser Gln Gln Pro Phe Val Val Thr Phe Phe	1253

05	235	240	245	
	AGG GCC AGT CCG AGT CCC ATC CGC ACC CCT CGG GCA GTG AGG CCA CTG			1301
	Arg Ala Ser Pro Ser Pro Ile Arg Thr Pro Arg Ala Val Arg Pro Leu			
	250	255	260	265
10	AGG AGG AGG CAG CCG AAG AAA AGC AAC GAG CTG CCG CAG GCC AAC CGA			1349
	Arg Arg Arg Gln Pro Lys Lys Ser Asn Glu Leu Pro Gln Ala Asn Arg			
		270	275	280
	CTC CCA GGG ATC TTT GAT GAC GTC CAC GGC TCC CAC GGC CGG CAG GTC			1397
	Leu Pro Gly Ile Phe Asp Asp Val His Gly Ser His Gly Arg Gln Val			
		285	290	295
15	TGC CGT CGG CAC GAG CTC TAC GTC AGC TTC CAG GAC CTC GGC TGG CTG			1445
	Cys Arg Arg His Glu Leu Tyr Val Ser Phe Gln Asp Leu Gly Trp Leu			
		300	305	310
	GAC TGG GTC ATC GCT CCC CAA GGC TAC TCG GCC TAT TAC TGT GAG GGG			1493
	Asp Trp Val Ile Ala Pro Gln Gly Tyr Ser Ala Tyr Tyr Cys Glu Gly			
20		315	320	325
	GAG TGC TCC TTC CCA CTG GAC TCC TGC ATG AAT GCC ACC AAC CAC GCC			1541
	Glu Cys Ser Phe Pro Leu Asp Ser Cys Met Asn Ala Thr Asn His Ala			
		330	335	340
	ATC CTG CAG TCC CTG GTG CAC CTG ATG AAG CCA AAC GCA GTC CCC AAG			1589
25	Ile Leu Gln Ser Leu Val His Leu Met Lys Pro Asn Ala Val Pro Lys			
		350	355	360
	GCG TGC TGT GCA CCC ACC AAG CTG AGC GCC ACC TCT GTG CTC TAC TAT			1637
	Ala Cys Cys Ala Pro Thr Lys Leu Ser Ala Thr Ser Val Leu Tyr Tyr			
		365	370	375
30	GAC AGC AGC AAC AAC GTC ATC CTG CGC AAA GCC CGC AAC ATG GTG GTC			1685
	Asp Ser Ser Asn Asn Val Ile Leu Arg Lys Ala Arg Asn Met Val Val			
		380	385	390
	AAG GCC TGC GGC TGC CAC TGAGTCAGCC CGCCAGCCC TACTGCAGCA			1733
35	Lys Ala Cys Gly Cys His			
		395		
	ATCACTGGC CGTCGTTTTA CAACGTGTGA CTGGAAAAC CCTGGCGTTA CCCAACTTAA			1793
	TCGCCTTGCA GCACATCCCC CTTTCGCCAG CTGGCTAATA GCGAAGAGGC CCCGCACCGA			1853
	TCGCCCTTCC CAACAGTTGC GCCCCAGTGA ATGGCGAATG GCAAATTGTA AGCGTTAATA			1913
	TTTTGTAAAA ATTTCGGTTA AATTTTTT			1941

(2) INFORMATION FOR SEQ ID NO:6:

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05 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 399 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (ix) FEATURE:
 (D) OTHER INFORMATION: /product= "hOP2-PP"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Thr Ala Leu Pro Gly Pro Leu Trp Leu Leu Gly Leu Ala Leu Cys
 1 5 10 15

15 Ala Leu Gly Gly Gly Pro Gly Leu Arg Pro Pro Pro Gly Cys Pro
 20 25 30

Gln Arg Arg Leu Gly Ala Arg Asp Arg Asp Val Gln Arg Glu Ile Leu
 35 40 45

20 Ala Val Leu Gly Leu Pro Gly Arg Pro Arg Pro Arg Ala Pro Pro Ala
 50 55 60

Ala Ser Arg Leu Pro Ala Ser Ala Pro Leu Phe Met Leu Asp Leu Tyr
 65 70 75 80

His Arg Met Ala Gly Asp Asp Asp Glu Asp Gly Ala Ala Glu Ala Leu
 85 90 95

25 Gly Arg Ala Asp Leu Val Met Ser Phe Val Asn Met Val Glu Arg Asp
 100 105 110

Arg Ala Leu Gly His Gln Glu Pro His Trp Lys Glu Phe Arg Phe Asp
 115 120 125

30 Leu Thr Gln Ile Pro Ala Gly Glu Ala Val Thr Ala Ala Glu Phe Arg
 130 135 140

Ile Tyr Lys Val Pro Ser Ile His Leu Leu Asn Arg Thr Leu His Val
 145 150 155 160

Ser Met Phe Gln Val Val Gln Glu Gln Ser Asn Arg Glu Ser Asp Leu
 165 170 175

35 Phe Phe Leu Asp Leu Gln Thr Leu Arg Ala Gly Asp Glu Gly Trp Leu
 180 185 190

Val Leu Asp Val Thr Ala Ala Ser Asp Cys Trp Leu Leu Lys Arg His
 195 200 205

Lys Asp Leu Gly Leu Arg Leu Tyr Val Glu Thr Glu Asp Gly His Ser
 210 215 220

05 Val Asp Pro Gly Leu Ala Gly Leu Leu Gly Gln Arg Ala Pro Arg Ser
 225 230 235 240
 Gln Gln Pro Phe Val Val Thr Phe Phe Arg Ala Ser Pro Ser Pro Ile
 245 250
 10 Arg Thr Pro Arg Ala Val Arg Pro Leu Arg Arg Arg Gln Pro Lys Lys
 260 265 270
 Ser Asn Glu Leu Pro Gln Ala Asn Arg Leu Pro Gly Ile Phe Asp Asp
 275 280 285
 Val His Gly Ser His Gly Arg Gln Val Cys Arg Arg His Glu Leu Tyr
 290 295 300
 15 Val Ser Phe Gln Asp Leu Gly Trp Leu Asp Trp Val Ile Ala Pro Gln
 305 310 315 320
 Gly Tyr Ser Ala Tyr Tyr Cys Glu Gly Glu Cys Ser Phe Pro Leu Asp
 325 330 335
 20 Ser Cys Met Asn Ala Thr Asn His Ala Ile Leu Gln Ser Leu Val His
 340 345 350
 Leu Met Lys Pro Asn Ala Val Pro Lys Ala Cys Cys Ala Pro Thr Lys
 355 360 365
 Leu Ser Ala Thr Ser Val Leu Tyr Tyr Asp Ser Ser Asn Asn Val Ile
 370 375 380
 25 Leu Arg Lys Ala Arg Asn Met Val Val Lys Ala Cys Gly Cys His
 385 390 395

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 102 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: protein
- (ix) FEATURE:
 - (A) NAME/KEY: Protein
 - (B) LOCATION: 1..102
 - (D) OTHER INFORMATION: /label= OPX
 /note= "WHEREIN EACH XAA IS INDEPENDENTLY SELECTED
 FROM A GROUP OF ONE OR MORE SPECIFIED AMINO ACIDS
 AS DEFINED IN THE SPECIFICATION
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

05 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 65 70 75 80
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Cys
 85 90 95

Xaa

10 (2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 102 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

15 (ii) MOLECULE TYPE: protein

(ix) FEATURE:

- (A) NAME/KEY: Protein
- (B) LOCATION: 1..102
- (D) OTHER INFORMATION: /label= PROTEIN

20 /note= "WHEREIN EACH XAA INDEPENDENTLY INDICATES
 ONE OF THE 20 NATURALLY-OCCURRING L-ISOMER A-AMINO
 ACIDS, OR A DERIVATIVE THEREOF."

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

25 Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1 5 10 15
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa
 20 25 30
 Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 35 40 45
 30 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 50 55 60
 Xaa Cys Cys Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 65 70 75 80
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 85 90 95
 Xaa Xaa Cys Xaa Cys Xaa
 100

- 05 What is claimed is:
1. A polypeptide chain comprising an amino acid sequence described by residues 303-399 of Seq. ID No. 5.
 - 10 2. The polypeptide chain of claim 1 comprising an amino acid sequence described by residues 297-399 of Seq. ID No. 5.
 3. The polypeptide chain of claim 2 comprising of amino acid sequence described by residues 267-399 of Seq. ID No. 5.
 - 15 4. The polypeptide chain of claim 3 comprising an amino acid sequence described by residues 264-399 of Seq. ID No. 5.
 5. The polypeptide chain of claim 4 comprising an amino acid sequence described by residues 240-399 of Seq. ID No. 5.
 - 20 6. The polypeptide chain of claim 5 comprising an amino acid sequence described by residues 1-399 of Seq. ID No. 5.
 7. A polypeptide chain comprising an amino acid sequence described by residues of 301-397 of Seq. ID No. 3.
 - 25 8. The polypeptide chain of claim 7 comprising an amino acid sequence described by residues 296-397 of Seq. ID No.3.

- 05 9. The polypeptide chain of claim 8 comprising an amino acid sequence described by residues 259-397 of Seq. ID No. 3.
- 10 10. The polypeptide chain of claim 9 comprising an amino acid described by residues 1-397 of Seq. ID No. 3.
- 15 11. A polypeptide chain useful as a subunit of a dimeric osteogenic protein comprising a pair of disulfide-bonded polypeptide chains, said polypeptide chain having an amino acid sequence described by residues 303-399 of Seq. ID No. 5, including allelic and species variants thereof, such that the dimeric osteogenic protein comprising said polypeptide chain has a conformation capable of inducing endochondral bone formation when implanted in a mammal in association with a matrix.
- 20 12. The polypeptide chain of claim 11 wherein said amino acid sequence comprises residues 261-399 of Seq. ID 5.
- 25 13. The polypeptide chain of claim 11 wherein the amino acid sequence comprises residues 301-397 of Seq. ID No. 3.
14. The polypeptide chain of claim 13 wherein said amino acid sequent comprises residues 259-397 of Seq. ID No. 3.
- 30 15. A dimeric osteogenic protein capable of inducing endochondral bone formation in a mammal when implanted in said mammal in association with a matrix;

- 05 said protein comprising a pair of disulfide-bonded
polypeptide chains constituting a dimeric species,
wherein each said polypeptide chain is the polypeptide
chain of claim 11.
- 10 16. The polypeptide chain of claim 3 or 11 produced by
expression of recombinant DNA in a host cell.
17. The polypeptide chain of claim 16 wherein said
host cell is a procaryotic host cell.
18. The polypeptide chain of claim 16 wherein said
host cell is a mammalian cell.
- 15 19. The polypeptide of claim 1, 3 or 11 that is
glycosylated.
20. A nucleic acid encoding the polypeptide chain of
claim 1, 3, or 11.
- 20 21. A dimeric protein comprising a pair of polypeptide
chains expressed from a DNA sequence described by ID
No. 3 or ID No. 5, including allelic and species
variants thereof, such that, when said polypeptide
chains are oxidized to produce a disulfide-bonded
dimeric species, the dimeric species has a conformation
25 that is capable of inducing endochondral bone or
cartilage formation when disposed within a matrix and
implanted in a mammal.

hOP2	Ala	Val	Arg	Pro	Leu	Arg	Arg	Arg
mOP2	...	Ala	Lys
					5			
hOP2	Gln	Pro	Lys	Lys	Ser	Asn	Glu	Leu
mOP2	Thr
		10					15	
hOP2	Pro	Gln	Ala	Asn	Arg	Leu	Pro	Gly
mOP2	...	His	Pro	...	Lys
				20				
hOP2	Ile	Phe	Asp	Asp	Val	His	Gly	Ser
mOP2	Gly
	25					30		
hOP2	His	Gly	Arg	Gln	Val	Cys	Arg	Arg
mOP2	Arg	Glu
			35					40
hOP2	His	Glu	Leu	Tyr	Val	Ser	Phe	Gln
mOP2	Arg	...	Arg
					45			
hOP2	Asp	Leu	Gly	Trp	Leu	Asp	Trp	Val
mOP2
		50					55	
hOP2	Ile	Ala	Pro	Gln	Gly	Tyr	Ser	Ala
mOP2
				60				
hOP2	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ser
mOP2	Ala
	65					70		

Fig. 1.1

hOP2	Phe	Pro	Leu	Asp	Ser	Cys	Met	Asn
mOP2
			75					80
hOP2	Ala	Thr	Asn	His	Ala	Ile	Leu	Gln
mOP2
					85			
hOP2	Ser	Leu	Val	His	Leu	Met	Lys	Pro
mOP2
		90					95	
hOP2	Asn	Ala	Val	Pro	Lys	Ala	Cys	Cys
mOP2	Asp	Val
				100				
hOP2	Ala	Pro	Thr	Lys	Leu	Ser	Ala	Thr
mOP2
	105					110		
hOP2	Ser	Val	Leu	Tyr	Tyr	Asp	Ser	Ser
mOP2
			115					120
hOP2	Asn	Asn	Val	Ile	Leu	Arg	Lys	Ala
mOP2	His
					125			
hOP2	Arg	Asn	Met	Val	Val	Lys	Ala	Cys
mOP2
		130					135	
hOP2	Gly	Cys	His					
mOP2					

Fig. 1.2

SUBSTITUTE SHEET

hOP1	Ser	Thr	Gly	Ser	Lys	Gln	Arg	Ser	Gln
mOP1	Gly
hOP2	Ala	Val	Arg	Pro	Leu	Arg	...	Arg	...
mOP2	Ala	Ala	Arg	Pro	Leu	Lys	...	Arg	...
	1				5				
hOP1	Asn	Arg	Ser	Lys	Thr	Pro	Lys	Asn	Gln
mOP1
hOP2	Pro	Lys	Lys	Ser	Asn	Glu	Leu	Pro	Gln
mOP2	Pro	Lys	Lys	Thr	Asn	Glu	Leu	Pro	His
	10					15			
hOP1	Glu	Ala	Leu	Arg	Met	Ala	Asn	Val	Ala
mOP1	Ser
hOP2	Ala	Asn	Arg	Leu	Pro	Gly	Ile	Phe	Asp
mOP2	Pro	Asn	Lys	Leu	Pro	Gly	Ile	Phe	Asp
		20					25		
hOP1	Glu	Asn	Ser	Ser	Ser	Asp	Gln	Arg	Gln
mOP1
hOP2	Asp	Val	His	Gly	...	His	Gly
mOP2	Asp	Gly	His	Gly	...	Arg	Gly	...	Glu
			30					35	
hOP1	Ala	Cys	Lys	Lys	His	Glu	Leu	Tyr	Val
mOP1
hOP2	Val	...	Arg	Arg
mOP2	Val	...	Arg	Arg
				40					45

Fig. 2.1

hOP1	Ser	Phe	Arg	Asp	Leu	Gly	Trp	Gln	Asp
mOP1
hOP2	Gln	Leu	...
mOP2	Arg	Leu	...
					10				
hOP1	Trp	Ile	Ile	Ala	Pro	Glu	Gly	Tyr	Ala
mOP1
hOP2	...	Val	Gln	Ser
mOP2	...	Val	Gln	Ser
	55					60			
hOP1	Ala	Tyr	Tyr	Cys	Glu	Gly	Glu	Cys	Ala
mOP1
hOP2	Ser
mOP2
		65					70		
hOP1	Phe	Pro	Leu	Asn	Ser	Tyr	Met	Asn	Ala
mOP1
hOP2	Asp	...	Cys
mOP2	Asp	...	Cys
			75					80	
hOP1	Thr	Asn	His	Ala	Ile	Val	Gln	Thr	Leu
mOP1
hOP2	Leu	...	Ser	...
mOP2	Leu	...	Ser	...
				85					90

Fig. 2.2

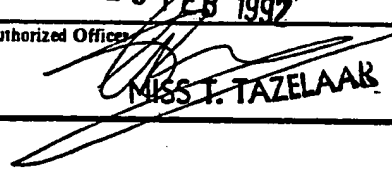
hOP1	Val	His	Phe	Ile	Asn	Pro	Glu	Thr	Val
mOP1	Asp
hOP2	Leu	Met	Lys	...	Asn	Ala	...
mOP2	Leu	Met	Lys	...	Asp	Val	...
					95				
hOP1	Pro	Lys	Pro	Cys	Cys	Ala	Pro	Thr	Gln
mOP1
hOP2	Ala	Lys
mOP2	Ala	Lys
	100					105			
hOP1	Leu	Asn	Ala	Ile	Ser	Val	Leu	Tyr	Phe
mOP1
hOP2	...	Ser	...	Thr	Tyr
mOP2	...	Ser	...	Thr	Tyr
		110					115		
hOP1	Asp	Asp	Ser	Ser	Asn	Val	Ile	Leu	Lys
mOP1	Asp
hOP2	...	Ser	...	Asn	Arg
mOP2	...	Ser	...	Asn	Arg
			120					125	
hOP1	Lys	Tyr	Arg	Asn	Met	Val	Val	Arg	
mOP1	
hOP2	...	Ala	Lys	
mOP2	...	His	Lys	
				130					
hOP1	Ala	Cys	Gly	Cys	His				
mOP1				
hOP2				
mOP2				
	135								

Fig. 2.3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 91/07635

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.C1.5	C 12 N 15/00	C 07 K 7/10
A 61 K 37/02	A 61 K 27/00	C 07 K 13/00
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.C1.5	C 07 K	A 61 K
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ^o	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X A	WO,A,8909788 (CREATIVE BIOMOLECULES) 19 October 1989, see the whole document ---	11,15-21 1-10,12-14
X A	WO,A,9011366 (GENETICS INSTITUTE) 4 October 1990, see the whole document ---	11,15-21 11,15-21
X	EMBO Journal, volume 9, no. 7, 1990, Oxford University Press (Eynsham, Oxford, GB) E. Ozkaynak et al.: "OP-1 cDNA encodes an osteogenic protein in the TGF-beta family", pages 2085-2093, see the whole article -----	11,15-21
<p>^o Special categories of cited documents :¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
31-01-1992	20 FEB 1992	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	 MISS J. TAZELAAR	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
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US 9107635

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		EP-A- 0411105	06-02-91
		JP-T- 3504736	17-10-91
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		WO-A- 9011366	04-10-90
CA-A- 2030518	29-09-90		
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